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In this first annual report we present our progress on two different areas, protein production and crystallization. We are working on expression and purification of Yop proteins and their complexes. In the first year we learnt reasons why some of the Yop proteins are not crystallizing. We are using a novel approach of making complexes with synthetic peptides or partner proteins to overcome this problem. In the case of LcrV, we are using the inherent property of LcrV forming a coiled coil complex with LcrG to crystallize the complex.

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Structural Studies on Toxins and Virulence Factors of *Yersinia pestis*
Annual Report for the Period ending June 2004

Introduction

The overall goal of this project is to determine the three-dimensional structures of proteins and virulence factors from *Yersinia pestis*. The organism *Yersinia pestis* is responsible for the plague and is a potential and emerging biowarfare threat. This organism has evolved a powerful method of delivering effectors into cells of the host immune system. The effectors act to completely circumvent the immune system. Understanding the molecular mechanism of this system is important to get an insight into the process of phagocytosis and inflammation. The effector proteins are secreted into the cells by a type III secretion system which involves a number of proteins. Some of them are actively involved in the delivery system and others are required for pore formation. In this proposal we will be determining the three-dimensional structures of proteins involved in the delivery system and some of the effector proteins. These proteins will be selected by a structural genomics approach. Information derived from this proposal will help in developing vaccines and therapeutics for the plague. It will also help in identifying genetically engineered threats. The goal is to study the three-dimensional structures of Yop proteins and their corresponding chaperones. By studying the three-dimensional structures of effector proteins we can get an insight into the mechanism of toxification of the cells which would eventually lead to design of inhibitors to block the toxicity.

Body

Purification and crystallization of YopH:

YopH is a key translocated effector of *Y. pestis* that exhibits tyrosine phosphatase activity. The 50 kDa YopH protein is organized into two modular domains separated by a linker region. The N terminal domain has phosphopeptide binding activity and functions as a substrate-targeting domain for the C-terminal catalytic domain. The two

separate domains of YopH have been crystallized and their structures solved (Smith *et al.*, 2001; Stuckey *et al.*, 1994). However, the structure of the native protein has not been determined. This is likely due to the fact that the native protein does not form crystals because of flexibility between the two domains. We believe that determining the structure of the native protein is important, as it will provide clues into potential functional interactions between the two domains. As part of this project we have undertaken studies to obtain the structure of the intact YopH protein (468 aa). A catalytically inactive form of YopH protein has been overexpressed with a C-terminal 6XHis tag in *E. coli* and purified by nickel affinity chromatography. The yield is typically 40 mg of protein per liter of culture volume.

Crystallization trials have been initiated using samples of the purified protein and still in progress. The failure to obtain crystallization condition so far may be because of the flexibility of the protein. We have developed a strategy to overcome the flexibility between the two domains using a synthetic peptide substrate with two phosphorylation sites that we hope will be simultaneously engaged by each domain, thereby imparting a rigid conformation to the protein (see Year 2 Plan below).

Preliminary structural characterization of YopB, YopD and YopK:

Delivery of secreted effectors such as YopH into host cells infected with *Y. pestis* is controlled by a set of secreted "translocator" proteins. Three proteins are required for the translocation process, LcrV, YopB and YopD. A fourth protein, YopK, appears to function as a negative regulator of translocation. The structure of a mutant form of LcrV has recently been determined (Derewenda *et al.*, 2004). However, the structures of YopB, YopD and YopK have not been determined. YopB (42 kDa) and YopD (33 kDa) contain predicted transmembrane domains and are thought to form a channel in the host cell membrane. YopK (21 kDa) does not have any recognizable features, and its function remains mysterious. Attempts to overexpress recombinant forms of YopB and YopD in *E. coli* have met with no success, due to their hydrophobic characteristics. As an alternative approach, we have engineered *Yersinia* to secrete these proteins into growth media, and we have investigated the possibility that the secreted forms of these proteins could be used for structural determinations. Currently we are working with relatively

small volumes of culture (less than 100 ml) and proteins secreted into *Yersinia* growth media are concentrated by filtration and analyzed by native polyacrylamide gel electrophoresis. Although yields of protein are small (e.g. 10 micrograms) this procedure does provide enough protein for initial studies. The results of these experiments showed that all three proteins, YopB, YopD and YopK, were secreted as soluble proteins. However, all three proteins migrated as multimeric forms on the gels. YopB and YopD formed multimeric ladders of bands on the gels, and YopK ran as a broad band at a very high molecular weight. Thus, although the secreted forms of YopB, YopD and YopK are soluble, the multimeric forms of these proteins will make protein crystallization and X-ray determination challenging. Preliminary results indicate that YopB and YopD are forming homo- and hetero-oligomers. We plan additional studies in Year 2 (see below) to determine if the hetero-oligomeric forms of YopB and YopD are suitable for crystallization studies. However, we are also trying to express YopB and YopD individually as membrane proteins. This work is in progress using a new protocol for expression developed by Dr. Studier, an expert in T7 expression system.

Expression and Crystallization of LcrV:

LcrV was expressed in BL21 cells. The protein was expressed with Se labeled methionines that will be useful for the structure determination. Protein expression and purification were optimized, and 60mg of homogeneous protein was obtained per litter of culture. Purified protein was used for crystallization. Since the protein is highly soluble, 10 – 40 mg/ml protein concentration was used for crystallization trials. The protein crystallization attempts showed little clue of any crystallization conditions. In the meantime the crystal structure of LcrV triple mutant has been reported (Derewenda *et al.*, 2004). It is perceived that the wild-type protein is recalcitrant to crystallization.

A mutation study on LcrV explains coiled-coil interaction of LcrV with LcrG (Lawton *et. al.*, 2002). This paper identified the coiled-coil region for both the proteins of LcrV and LcrG. The coiled-coil region identified in LcrG is D7 through K28 and this region is presumed to interact with LcrV to form a complex. We will be using this fact to crystallize the complex (see Year 2 plan).

In the meantime optimizing the expression protocol of LcrG protein is in progress. Once we get LcrG protein we plan to make LcrV-LcrG complex. This will be more effective and useful for the project.

Crystallization of SycD:

SycD is the intrabacterial chaperone of YopD that directs the translocation of the secreted Yop effector proteins across the target membrane. Crystallization of this protein, SycD is also in progress. Expression and purification of this protein is successful and the initial crystallization trials are in progress.

Key Research Accomplishments: None yet.

Reportable outcomes

None as of now.

Conclusions

In our studies we have found reasons for difficulties in expressing some of Yop proteins and in crystallizing them. We will be using this information in the second year to overcome these difficulties.

Year 2 plans

YopH-peptide substrate complex:

Two synthetic peptide substrates based on a natural substrate (Cas) of YopH have been synthesized. The sequences of these peptides are shown below, with phosphorylated tyrosines (pY) shown in bold:

Cas peptide (342-371): GSQDI**pY**DVPPVRGLLPNQYGQEV**pY**DTPPMA

Cas peptide (258-287): PATDL**pY**QVPPGPGSPAQDI**pY**QVPPSAGTGH

These peptides will be individually mixed with purified inactive YopH and crystallization studies will be initiated. If the substrate binding sites of the N-terminal domain and the catalytic domain are in close proximity in the native protein, we anticipate that the peptides will form a bridge between the two domains and create a rigid conformation in the protein that will facilitate crystallization.

YopB-YopD complex:

Preliminary studies indicate that YopB and YopD are secreted into growth media by *Yersinia* in a soluble form. These proteins appear to be forming homo- and hetero-oligomeric complexes. It is possible that the hydrophobic regions of these proteins are buried in the oligomers, which allows them to be soluble in aqueous solutions. Although the homo-oligomeric forms of these proteins may not be suitable for crystallization, we anticipate that the hetero-oligomeric complexes may represent distinct species that could be isolated in pure form and used for crystallization studies. To investigate this possibility, we will purify a secreted form of YopB that contains an N-terminal 6X his tag. We have confirmed that this tagged form of YopB retains full biological activity. The purified YopB will be run on native gels to determine the percentage of the protein that is in a homo- or hetero-oligomeric form. If we are able to identify a specific YopB-YopD complex on native gels, we will then attempt to obtain sufficient amounts of this complex for structural studies.

LcrV-peptide complex:

LcrG interacts with LcrV through a N-terminal domain in LcrG. We have purchased a synthetic peptide of D7 through K28 (22 amino acids) and have made LcrV-peptide complex. The idea is that the flexible region/residues of LcrV that hiders the crystallization process might be stabilized by the peptide interaction and a stable protein-peptide complex will be formed. Crystallization of the LcrV-peptide complex is in progress.

Personnel in the Project

1. S. Swaminathan (PI)	Scientist	30% effort
2. S. Eswaramoorthy	Associate Scientist	40% effort
3. R. Agarwal	Research Associate	100% effort

Sub-contract to State University of New York at Stony Brook

1. J. Bliska	Professor	10% effort
2. M. Ivanov	Technician	100% effort

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